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Functional importance of calcium binding sites in outer membrane phospholipase A

Roelie L. Kingma [a], Harm J. Snijder [b], Bauke W. Dijkstra [b], Niek Dekker [a,1], Maarten R. Egmond [a,*]

[a] *Department of Membrane Enzymology, Centre for Biomembranes and Lipid Enzymology, Institute of Biomembranes, Utrecht University, P.O. Box 80054, 3508 TB Utrecht, Netherlands*

[b] *Laboratory of Biophysical Chemistry, Department of Chemistry, University of Groningen, Groningen, Netherlands*

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Abstract

Outer membrane phospholipase A (OMPLA) is an integral membrane enzyme that hydrolyses phospholipids requiring Ca^{2+} as cofactor. In vitro studies have shown that OMPLA is only active as a dimer. The structures of monomeric and dimeric OMPLA provided possible clues to the activation process. In the inhibited dimeric species calcium ions are located at the dimer interface ideally suited to stabilise the oxyanion intermediates formed during catalysis. The side chain hydroxyl function of Ser152 is one of the ligands of this interfacial calcium. In the crystal structure of monomeric OMPLA the interfacial calcium site is lacking, but calcium was found to bind at a site involving the carboxylates of Asp149 and Asp184. In the current study the relevance of the identified calcium sites has been studied by site-directed mutagenesis. The Ser152Asn variant confirmed the importance of the interfacial calcium site for catalysis, and also demonstrated that this site is essentially involved in the dimerisation process. Replacements of the ligands in monomeric OMPLA, i.e. Asp149Asn, Asp149Ala and Asp184Asn, only showed minor effects on catalytic activity and dimerisation. A stronger effect observed for the variant Asp184Ala was explained by the proximity of Asp184 to the catalytically important Ser152 residue. We propose that Asp149 and Asp184 provide an electronegative funnel that may facilitate Ca^{2+} transfer to the interfacial calcium site.

Keywords: Outer membrane phospholipase A; Calcium binding; Catalysis; Membrane enzyme

1. Introduction

The outer membrane phospholipase A (OMPLA) is a 31 kDa integral membrane that hydrolyses acyl ester bonds in phospholipids and lysophospholipids, requiring calcium as an essential cofactor [1]. OMPLA is encoded by the *pldA* gene that is widespread among Gram-negative bacteria, suggesting an important function. The physiological function has been studied most extensively in the *Escherichia coli* enzyme, where it participates in the secretion of colicins

Abbreviations: C_{12}SB, dodecyl-*N*,*N*-dimethyl-1-ammonio-3-propanesulphonate; C_{16}PC, hexadecanoylthioethane-1-phosphocholine; C_{16}PN, hexadecylphosphocholine; OMPLA, outer membrane phospholipase A; SDS–PAGE, sodium dodecyl sulphate–polyacrylamide gel electrophoresis

* Corresponding author. Fax: +31-30-252-2478.

E-mail address: m.r.egmond@chem.uu.nl (M.R. Egmond).

[1] Present address: AstraZeneca R&D, Structural Chemistry Laboratory, S-431 83 Mölndal, Sweden.

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[2,3]. Moreover, OMPLA has been implicated as virulence factor in bacterial pathogenicity. In *Campylobacter coli*, OMPLA was identified as a major haemolytic factor [4], while *Helicobacter pylori* OMPLA is involved in the invasion of gastric mucosa [5]. Nevertheless, *E. coli* strains lacking the *pldA* gene grow normally and have a normal phospholipid composition.

Whereas in normal growing cells OMPLA activity is dormant, the enzyme becomes active in cells with a perturbed cell envelope structure, e.g. in *E. coli* cells with a 'fad' or an 'envC' mutation [6,7]. OMPLA activity can also be induced in wild-type cells by phage-induced lysis [8], heat shock [9] or colicin secretion [3,10], which are also processes that disrupt the integrity of the outer membrane.

It has been shown that in vitro activity is regulated by reversible dimerisation [11]. Chemical cross-linking on whole cells demonstrated that OMPLA is present in the outer membrane as a monomeric species, and dimerisation occurs when activation is triggered, e.g. by the action of bacteriocin-release protein [3]. This suggests that also in vivo activity is regulated by dimerisation.

Recently, the crystal structures of monomeric and dimeric forms of OMPLA were solved [12]. The OMPLA monomer consists of a 12-stranded antiparallel β-barrel with long extracellular loops and short periplasmic turns, similar to other outer membrane protein structures solved to date. The barrel has a flat and a convex side, with a hydrophobic outer surface embedded in the outer membrane. The active site, consisting of the previously identified Ser144 [13,14], His142 [15] and Asn156 [16], is located at the hydrophilic/hydrophobic boundary of the outer leaflet of the outer membrane. In the dimeric structure, two monomers associate without major conformational changes with their flat sides facing each other. The necessity for dimerisation to obtain active protein becomes obvious as both subunits in the dimer are required to form two functional active sites, substrate binding pockets and oxyanion holes.

In dimeric OMPLA a calcium site is present at the dimer interface [12]. The calcium site is formed by interactions of both monomers within the dimer. An involvement in oxyanion stabilisation has been proposed for this calcium ion [17].

Calcium soaking experiments with crystals of monomeric OMPLA revealed binding of calcium between two carboxylates in the extracellular loops L3 and L4. Thus, the inactive monomeric and inhibited dimeric species have different calcium binding sites. In this study, we have investigated the biochemical relevance of these two calcium sites using site-directed mutagenesis.

2. Materials and methods

2.1. Chemicals

Restriction enzymes and DNA modifying enzymes were obtained from New England Biolabs. Oligonucleotides were purchased from Pharmacia. Research grade dodecyl-*N*,*N*-dimethyl-1-ammonio-3-propanesulphonate (C_{12}SB) was obtained from Fluka and was purified by running a concentrated solution of the detergent in methanol/chloroform (1:1) through a column packed with Al_2O_3 to remove acidic impurities present in the commercial preparation. The solvent was evaporated under vacuum. Hexadecylphosphocholine (C_{16}PN) was synthesised as described by van Dam-Mieras et al. [18]. The substrate 2-hexadecanoylthioethane-1-phosphocholine, used for the OMPLA assay, was synthesised according to Aarsman et al. [19]. Radioactive $^{45}CaCl_2$ was purchased from Amersham. All other chemicals were of the highest purity commercially available.

2.2. Construction, expression and purification of variants

The Ser152Asn variant was constructed by Brok et al. [14]. pRK21 encodes wild-type OMPLA and was constructed by introduction of silent mutations into pRK2 [16] using the QuikChange site-directed mutagenesis method (Stratagene), hereby creating new unique restriction sites within the *pldA* gene. Using these sites, the codon of interest (Asp149 or Asp184) could be changed by cassette mutagenesis. The oligonucleotide duplexes encoding the mutations contained a new restriction site to facilitate screening. The nucleotide sequences were verified by DNA sequencing.

The proteins were expressed in BL21(DE3) using the T7 expression system. Cells of a 1 l culture were

induced with 0.4 mM isopropyl β-D-thiogalactopyranoside at an OD_{600} of 0.8. This resulted in the accumulation of protein in inclusion bodies. The inclusion bodies were isolated as described by Dekker et al. [20], subsequently dissolved in 8 M urea/25 mM glycine, pH 8.3, and folded by rapid 5-fold dilution into 1.4 M urea, 5 mM glycine, pH 8.3 and 2% octyl polyethylene glycol. Further purification was carried out as described by Kingma et al. [16]. After purification, the protein was loaded onto a Q-Sepharose column to replace the detergent C_{12}SB with C_{16}PN. After washing with 10 mM C_{16}PN/20 mM Tris–HCl, pH 8.3, and with 1 mM C_{16}PN/Tris–HCl, pH 8.3, the protein was eluted with 1 M KCl in 1 mM C_{16}PN/20 mM Tris–HCl, pH 8.3. The protein was dialysed twice against 10 vols. of 1 mM C_{16}PN/20 mM Tris–HCl, pH 8.3, and stored at 4°C.

2.3. Activity assay

OMPLA activities were determined spectrophotometrically using hexadecanoylthioethane-1-phosphocholine as a substrate. OMPLA was incubated overnight at a concentration of 0.05 mg/ml in buffer (20 mM Tris–HCl, pH 8.3, 2 mM EDTA, 1 mM C_{16}PN). Routinely, 50 ng of protein were assayed for enzymatic activity in 1 ml of assay buffer (50 mM Tris–HCl, pH 8.3, 5 mM $CaCl_2$, 0.2 mM Triton X-100, 0.1 mM dithio-bis(2-nitrobenzoic acid), 0.25 mM substrate). Initial velocities were calculated from the increase in absorbance at 412 nm. One unit corresponds with the conversion of 1 μmole of substrate per minute.

2.4. Effect of Ca^{2+} binding upon kinetics

Kinetic Ca^{2+} binding constants were determined using the aforementioned assay with minor modifications. Instead of 5 mM $CaCl_2$, 10 μM of EDTA was added to the assay buffer. Subsequently, calcium was titrated to the assay buffer and calcium binding constants were determined using unweighted non-linear regression fitting.

2.5. Equilibrium gel filtration

A column (50×1 cm) of Sephadex G-25 was equilibrated with buffer (20 mM Tris–HCl, pH 8.3; 100 mM KCl; 1 mM C_{16}PN) containing $^{45}CaCl_2$ of the desired concentration (equilibration buffer). Subsequently, the column was loaded with 0.6 ml protein (2–5 mg/ml) incubated in the equilibration buffer with an excess of $^{45}CaCl_2$ solution (2 mM). The protein was eluted with the equilibration buffer. Fractions of 350 μl were collected. 150 μl were transferred to scintillation vials and 4 ml of scintillation liquid were added to each vial. Radioactivity was determined in a Canberra liquid scintillation counter. Protein concentrations were determined spectrophotometrically using an $OD_{280}^{1\%}$ of 29.2. The obtained data were analysed according to Scatchard [21].

2.6. Glutaraldehyde cross-linking

OMPLA was incubated overnight at 0.2 mg/ml in buffer (50 mM HEPES, pH 8.3, 100 mM KCl, 3 mM C_{12}SB, and either 20 mM $CaCl_2$ or 20 mM EDTA) in a total volume of 100 μl. Subsequently, 5 μl of 1% glutaraldehyde in 2.5 mM C_{12}SB were added. The reaction was allowed to continue for 4 h at room temperature. Subsequently, 100 μl of gel loading buffer (0.1 M Tris–HCl, pH 6.8, 3% sodium dodecyl sulphate (SDS), 15.4% glycerol, 7.7% β-mercaptoethanol and 0.008% bromphenol blue) were added, and 20 μl of this solution (corresponding to 2 μg of OMPLA) were analysed by SDS–polyacrylamide gel electrophoresis (PAGE). Visualisation of the bands was achieved by staining with Coomassie brilliant blue.

3. Results

3.1. Construction, expression and purification of OMPLA variants

Fig. 1A shows the crystal structure of inhibited, dimeric OMPLA with one calcium ion per monomer bound at the dimer interface [12]. The calcium ion is coordinated by the hydroxyl group of Ser152 and the Ser106 carbonyl oxygen atom of one monomer, the Arg147 carbonyl oxygen atom of the other monomer, and three water molecules (Fig. 1B). Of these six coordinating groups, Ser152 is the only residue of which site-directed mutagenesis is expected to influence calcium binding. An earlier study showed that only Thr and Asn could functionally substitute for Ser152 with

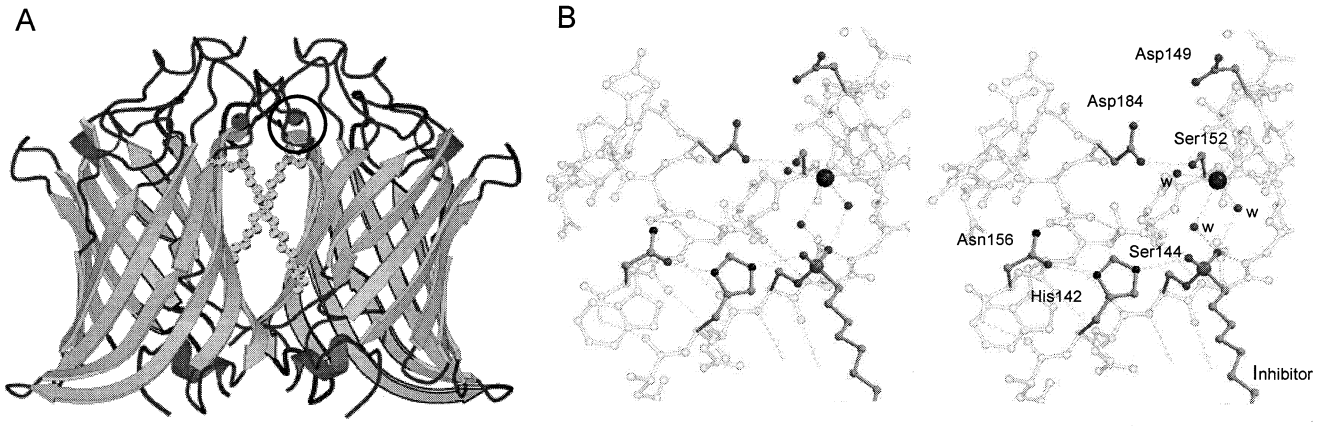

Fig. 1. (A) Crystal structure of the inhibited, dimeric OMPLA species with the interfacial calcium binding site encircled. The inhibitor is represented in a ball-and-stick fashion. (B) Stereo-view of the interfacial Ca^{2+} site in detail. The calcium ion is depicted in dark grey. Furthermore, the active site residues, the inhibitor and residues coordinating the calcium ion are highlighted (w represents water molecules). The calcium ion is in close proximity to the active site (at a distance of approx. 6 Å from Ser144Oγ). Interactions between atoms are depicted in dashed lines.

respectively 40% and 4% residual activity, whilst all other replacements yielded inactive enzyme [14]. The Ser152Asn variant was chosen to monitor calcium binding at the interfacial calcium site.

The structure of monomeric OMPLA with bound calcium is shown in Fig. 2. The Ca^{2+} is bound between a carboxylate oxygen atom of Asp 149 in loop 3 and a carboxylate oxygen atom of Asp184 in loop 4. To determine the contribution of this Ca^{2+} site to catalysis and dimerisation, Asp149 and Asp184 were replaced by either asparagine or alanine residues.

The proteins were expressed under control of a T7 promoter without a signal sequence resulting in the formation of approx. 250 mg of inclusion bodies per litre of bacterial culture. Expression levels were comparable for wild-type and variant OMPLAs. The inclusion bodies were folded in vitro with an efficiency of 50–60%, comparable to wild-type. After purifica-

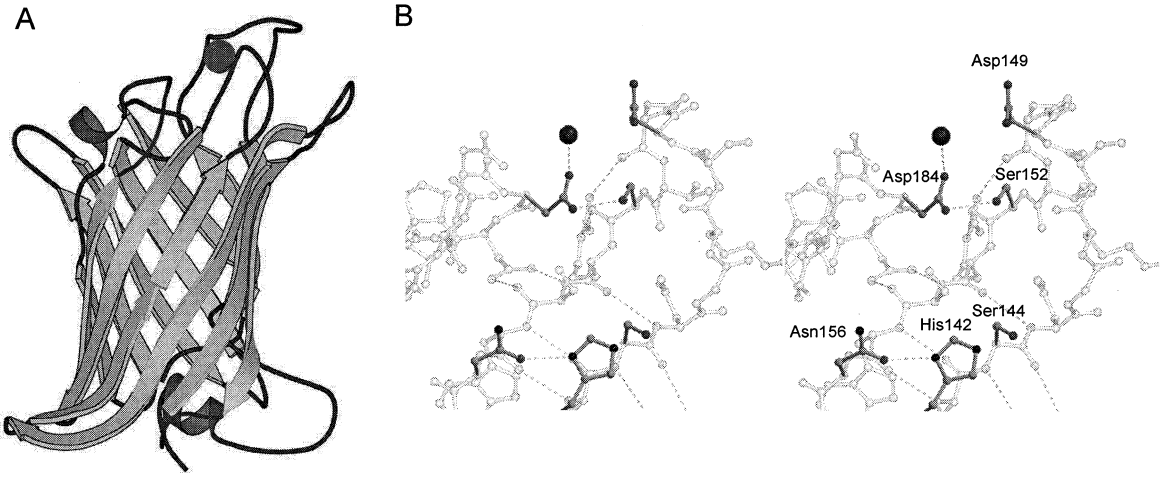

Fig. 2. (A) Structure of monomeric OMPLA with a calcium ion bound between loop 3 and loop 4. (B) Focus on the monomeric L3L4 Ca^{2+} site in stereo-view. The calcium ion is depicted in dark grey and the active site and residues important in calcium binding or hydrogen bonding interactions are highlighted. Interactions between atoms are represented as dashed lines. Water molecules are suggested to complete the calcium ligand sphere but could not be identified in the crystal structure likely due to high crystallographic B-factors in the loop regions.

tion approx. 40 mg of protein were obtained from 1 l of bacterial culture with a purity higher than 95%. To assess folding of the variants, migration behaviour on SDS–PAGE was determined. The β-barrel structure of OMPLA is highly stable, such that no denaturation occurs in the presence of SDS. The β-barrel only unfolds after boiling of the samples in SDS. The folded OMPLA species migrates faster on SDS–PAGE than does the fully unfolded form. This characteristic is called heat modifiability and can be used as an indicator for proper folding. All proteins displayed heat modifiability, indicating that they were correctly folded.

3.2. Enzymatic activities and calcium binding affinities in the kinetic assay

The presence of the cofactor calcium and its concentration are major determinants for enzymatic activity. In the presence of EDTA no enzymatic activity could be detected. The actual increase in activity upon addition of calcium depends on the type and concentration of detergent used to solubilise OMPLA. The detergent used in this study is the zwitterionic lysophospholipid analogue C_{16}PN [22]. In Fig. 3A the enzymatic activities for wild-type and variant OMPLAs are summarised. Wild-type OMPLA displays an activity of 84 U/mg in C_{16}PC, the substrate used in the standard assay. No effect on maximum activity was observed for Asp149Asn, Asp149Ala and the Asp184Asn variant. The Asp184Ala variant displayed a reduction in activity to 26 U/mg. The strongest effect was noted for the Ser152Asn variant yielding residual activity of 3 U/mg.

Upon titration with calcium in the kinetic assay a hyperbolic saturation curve was obtained from which apparent calcium affinity constants could be derived. The data are represented graphically in Fig. 3B. For wild-type OMPLA a value of 19 (±2.5) μM was found. The Asp149Ala displayed similar Ca^{2+} affinity, whereas the Asp149Asn variant showed increased affinity for calcium of 4.3 (±0.5) μM. Calcium binding of the Asp184Asn and Asp184Ala variants was severely affected with affinity constants of 0.43 (±0.03) and 2.7 (±0.6) mM, respectively. Severely impaired calcium affinity was also noted for the Ser152Asn variant with an affinity constant of 1.1 (±0.25) mM.

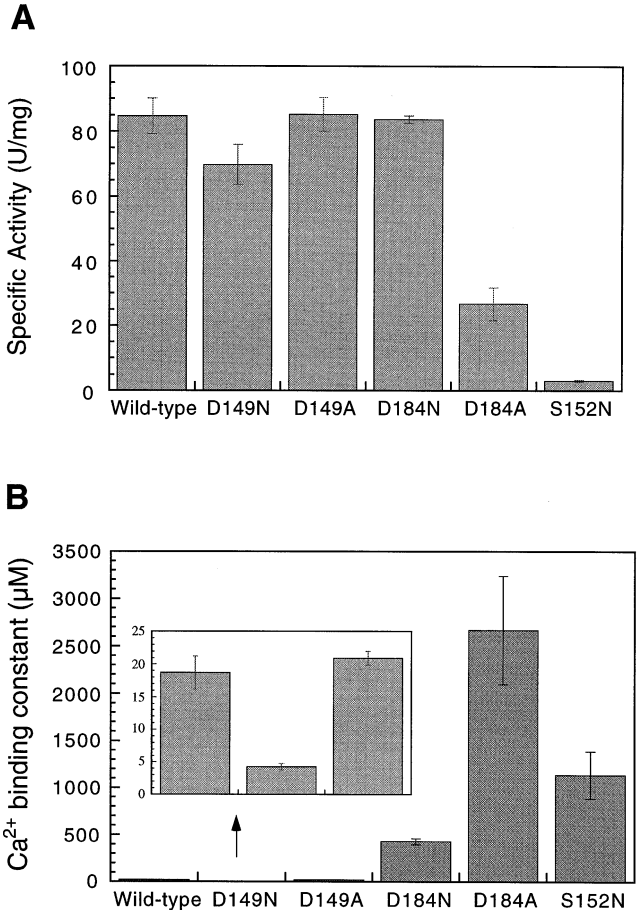

Fig. 3. Specific activities (A) and apparent calcium binding constants (B) of wild-type and variant OMPLAs as determined in the kinetic assay. Note that the inset in panel B represents values with 120-fold lower calcium affinity constants.

3.3. Calcium binding determined by gel filtration

Direct binding of calcium to OMPLA was studied using gel filtration with $^{45}Ca^{2+}$ under equilibrium conditions. The amount of calcium bound to OMPLA was determined by measuring the excess of radioactivity co-eluting with the protein relative to the level of radioactivity in buffer not containing protein. The experiment was repeated at various calcium concentrations. Reliable results were generally obtained up to a calcium concentration of 700 μM. The results were analysed as described by Ubarretxena-Belandia et al. [22], while stoichiometries and affinities were determined from hyperbolic binding curves using nonlinear regression. The results are summarised in Table 1. In wild-type OMPLA, one calcium ion was bound per OMPLA subunit with an affinity of 13

Table 1
Stoichiometries and apparent calcium binding constants as determined by gel filtration equilibrium experiments. n_1 and n_2 correspond with stoichiometries derived from a Scatchard plot, whereas $K_{Ca(1)}$ and $K_{Ca(2)}$ represent the corresponding calcium binding constants.

	n_1	$K_{Ca(1)}$ (μM)	n_2	$K_{Ca(2)}$ (μM)
Wild-type	0.9 ± 0.1	13 ± 4.5	2.7 ± 0.2	347 ± 57
Asp149Asn	1.1 ± 0.1	46 ± 6	2.0 ± 0.2	229 ± 69
Aps149Ala	1.2 ± 0.1	143 ± 28	–	–
Asp184Asn	1.3 ± 0.25	334 ± 105	–	–
Asp184Ala	–	–	–	–
Ser152Asn	1.5 ± 0.4	460 ± 138	–	–

μM. Furthermore, a second calcium was found to be bound with a much lower affinity of 347 μM. These data demonstrate that dimeric OMPLA can bind four calcium ions. In the Asp149Asn variant similar stoichiometry and affinity constants were obtained for the high affinity site, whereas the second site showed slightly improved affinity for calcium relative to wild-type OMPLA. For the Asp149Ala variant the affinity of the catalytic calcium site had decreased 10-fold. Under our experimental conditions, binding of another calcium ion was not observed. In the Asp184Asn variant, only one calcium ion was bound with modest affinity. Although the Asp184Ala variant was found to bind some calcium, the dissociation constant could not be properly determined.

For the representative variant of the interfacial calcium site, Ser152Asn, a stoichiometry value was found of 1.5 with an affinity of 460 μM. This stoichiometry and affinity may represent the average of two low affinity binding sites.

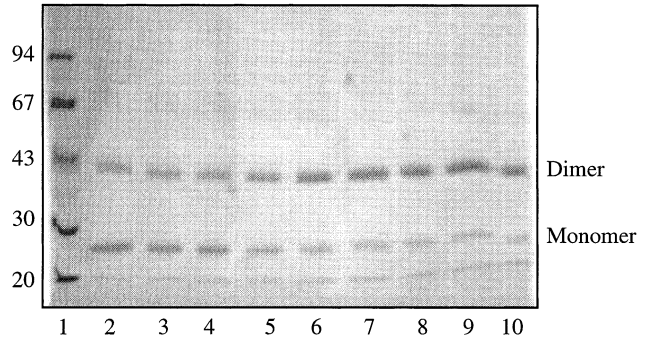

Fig. 4. Glutaraldehyde cross-linking of OMPLA as a function of calcium. The results are shown at various concentrations of calcium. Lanes: 1, molecular mass markers; 2, 2 mM EDTA; 3–10, 10, 20, 40, 100, 500, 1000, 5000 μM Ca^{2+}, respectively.

3.4. Calcium-dependent dimerisation

Chemical cross-linking was used to determine the involvement of the interfacial and L3L4 calcium site in dimerisation. OMPLA was incubated at various calcium concentrations and analysed by SDS–PAGE after addition of cross-linking reagent. In the presence of EDTA, the enzyme migrates mainly as a monomer after cross-linking (Fig. 4, lane 2). At elevated calcium concentrations, increasing amounts of protein were cross-linked into the dimeric form (Fig. 4, lanes 2–8). The affinity constant for calcium-dependent dimerisation was well below 100 μM (Fig. 4, lane 6 corresponds with 100 μM Ca^{2+}). However, also calcium-independent dimerisation was observed in the detergent $C_{16}PN$ (Fig. 4, lane 2) which hindered quantification of dimer fractions in dimerisation-affected variants. To overcome this problem we only determined calcium-dependent dimerisation in a qualitative manner for the variants. In the presence of EDTA, all proteins migrated mainly as a monomer similar to wild-type OMPLA (Fig. 5, lanes 3, 5, 7 and 9). However, at 20 mM calcium only the Ser152Asn did not migrate as a dimer and thus was

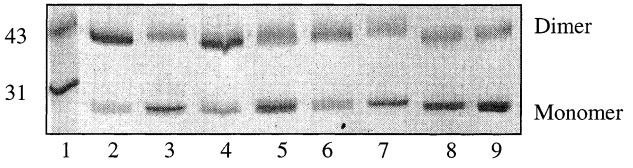

Fig. 5. Calcium-dependent glutaraldehyde cross-linking of wild-type and variant OMPLAs. Lanes: 1, molecular mass markers; 2 and 3, wild-type OMPLA; 4 and 5, Asp149Ala variant; 6 and 7, Asp184Ala variant; 8 and 9, Ser152Asn variant. Samples in the even lanes were cross-linked in the presence of 20 mM calcium, whereas the samples in the odd lanes were cross-linked in the presence of 2 mM EDTA.

affected in its capacity to dimerise (Fig. 5, lane 8). Overall, our studies suggest that the interfacial calcium site is essential for dimerisation, whereas the L3L4 site is not required for in vitro dimerisation.

4. Discussion

The presence of the cofactor calcium is essential for the enzymatic activity of OMPLA [23]. Biochemical studies revealed the presence of two distinct calcium binding sites, one with high affinity (36 μM) and the other with 10-fold lower affinity [22]. In agreement with these data, recent crystallographic studies revealed two Ca^{2+} binding sites in OMPLA [17]. One Ca^{2+} site was identified only in the structure of monomeric OMPLA (L3L4 site), whereas the other Ca^{2+} site was only found in the dimeric enzyme (interfacial site). In dimeric OMPLA no calcium binding was observed at the L3L4 site. In the present study, the involvement of the two Ca^{2+} sites in catalysis and dimerisation was studied in detail using site-directed mutagenesis.

The L3L4 and interfacial calcium sites are separated by only 6 Å. This close proximity requires a careful interpretation of calcium ligand replacements at either of the two sites. The interfacial calcium is shared between the two OMPLA monomers within the dimer and is coordinated by Ser152, two backbone carbonyl groups and three water molecules. Since the dimeric species of OMPLA is the active species, this calcium site was proposed to be the one essential for catalysis [12]. The Ser152Asn variant displayed 50-fold lower calcium affinity than wild-type OMPLA in the kinetic assay, as well as a 35-fold lower catalytic turnover, demonstrating that the interfacial site is indeed required for catalysis. Chemical cross-linking experiments indicate that even in the presence of excess calcium the Ser152Asn variant is present mainly as a monomer. This indicates that the interfacial calcium is not only involved in catalysis, but also in dimerisation of OMPLA.

In contrast, Asp149 is located at the periphery of the protein at 6 Å from the interfacial calcium site. Hence, mutations of Asp149 are expected to affect only L3L4 calcium binding. Both the Asp149Asn and Asp149Ala variants have a maximum specific activity and calcium affinity comparable to wild-type OMPLA, showing that calcium binding at the L3L4 site is indeed not important for OMPLA activity. Furthermore, calcium-dependent dimerisation was still observed, excluding the involvement of the L3L4 calcium in dimerisation.

The results of the Asp184 variants are more difficult to interpret as this residue is located in between the interfacial and L3L4 calcium site. Asp184 is not only a direct ligand of the L3L4 calcium site, but its carboxylate group is also within hydrogen bonding distance from the Ser152 hydroxyl group. Whereas the maximum specific activity of the Asp184Asn variant reaches wild-type levels of activity, the affinity for calcium is severely decreased. This effect is even more drastic for the Asp184Ala variant where not only calcium binding but also catalytic turnover is affected. We propose that Asp184 influences the strength of the interaction of the Ser152 hydroxyl group with the interfacial calcium ion.

In conclusion, the interfacial Ca^{2+} site is essential for both catalysis and calcium-dependent dimerisation, whereas the L3L4 site is redundant in vitro. These results are in accordance with sequence homology noted for several OMPLAs, revealing absolute conservation of Ser152, whereas Asp149 and Asp184 are not conserved in *H. pylori* (both Asp149 and Asp184), *Bordetella pertussis* (both Asp149 and Asp184), and *Neisseria* species (Asp149). It is noteworthy that in *H. pylori* OMPLA, which lacks the L3L4 calcium site, activity still depends on the presence of the cofactor calcium (Tannæs, unpublished data).

Whereas the L3L4 calcium is redundant in vitro, in vivo the local electronegative potential induced by Asp149 and Asp184 may be important to provide OMPLA with a low affinity calcium entry into the interfacial site. Further studies are required to clarify this possible function.

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